Elucidation of the Burkholderia cenocepacia hopanoid biosynthesis pathway uncovers functions for conserved proteins in hopanoid-producing bacteria

Schmerk, C. L., Welander, P. V., Hamad, M. A., Bain, K. L., Bernards, M. A., Summons, R. E., & Valvano, M. A. (2015). Elucidation of the Burkholderia cenocepacia hopanoid biosynthesis pathway uncovers functions for conserved proteins in hopanoid-producing bacteria. *Environmental Microbiology*, *17*(3), 735-750. https://doi.org/10.1111/1462-2920.12509

Published in:
Environmental Microbiology

Document Version:
Early version, also known as pre-print

Elucidation of the *Burkholderia cenocepacia* hopanoid biosynthesis pathway uncovers functions for conserved proteins in hopanoid-producing bacteria

Crystal L. Schmerk,[1] Paula V. Welander,[2] Mohamad A. Hamad,[1] Katie L. Bain,[1] Mark A. Bernards, [3] Roger E. Summons,[4] Miguel A. Valvano[1,5*]

[1] *Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, N6A 5C1, Canada.*
[2] *Department of Environmental Earth System Science, Stanford University, Stanford, California, United States.*
[3] *Department of Biology, University of Western Ontario, London, Ontario, N6A 5C1, Canada.*
[4] *Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States.*
[5] *Centre for Infection and Immunity, Queen's University Belfast, Belfast, BT9 5AE, United Kingdom.*

Running title: Hopanoid Biosynthesis in *B. cenocepacia* K56-2

* For correspondence. E-mail: m.valvano@qub.ac.uk; Tel. (+44) 28 9097 2878; Fax: (+44) 28 9097 2671.

Summary

Hopanoids are bacterial surrogates of eukaryotic membrane sterols and among earth's most abundant natural products. Their molecular fossils remain in sediments spanning more than a billion years. However, hopanoid metabolism and function are not fully understood. ***Burkholderia*** **species are environmental opportunistic pathogens that produce hopanoids and also occupy diverse ecological niches. We investigated hopanoids biosynthesis in** ***B. cenocepacia*** **by deletion mutagenesis and structural characterization of the hopanoids produced by the mutants. The enzymes encoded by** ***hpnH*** **and** ***hpnG*** **were essential for production of all C_{35} extended hopanoids, including bacteriohopanetetrol (BHT), BHT glucosamine and BHT cyclitol ether. Deletion of** ***hpnI*** **resulted in BHT production, while Δ*hpnJ*** **produced only BHT glucosamine. Thus, HpnI is required for BHT glucosamine production while HpnJ is responsible for its conversion to the cyclitol ether. The Δ*hpnH*** **and Δ*hpnG*** **mutants could not grow under any stress condition tested, whereas Δ*hpnI*, Δ*hpnJ*, and Δ*hpnK*** **displayed wild type growth rates when exposed to detergent, but varying levels of sensitivity to low pH and polymyxin B. This study not only elucidates the biosynthetic pathway of hopanoids in** ***B. cenocepacia*****, but also uncovers a biosynthetic role for the conserved proteins HpnI, HpnJ, and HpnK in other hopanoid-producing bacteria.**

Keywords: hopanoids/*Burkholderia cenocepacia*/membrane permeability

Introduction

Hopanoids are bacterial membrane lipids ubiquitous in modern day environments dominated by microbes and readily preserved in ancient sedimentary rock (Ourisson and Albrecht, 1992), making them ideal tools for reconstructing and understanding microbial ecosystems (Brocks et al., 2004; Summons and Lincoln, 2012). Due to size and structural similarities, hopanoids are considered bacterial surrogates of eukaryotic sterols, possibly regulating the fluidity and permeability of the bacterial membrane (Ourisson et al., 1987; Kannenberg, 1999). Only recently have studies focused on determining the biological function of hopanoids using genetic manipulation and *in vivo* experimentation. Such studies confirm that hopanoids are involved in enhancing the stability and impermeability of the bacterial membrane, conferring resistance to multiple stress conditions including pH, temperature, and exposure to detergents and antibiotics (Doughty et al., 2009; Welander et al., 2009; Schmerk et al., 2011; Malott et al., 2012). However, it remains unclear what functional importance can be ascribed to particular variations in the hopanoid core and side chain structures.

Hopanoids in lower eukaryotes and bacteria are derived from the direct cyclization of squalene (Rohmer et al., 1984; Ochs et al., 1992), which results in a pentacyclic triterpenoid core commonly modified by unsaturation, methylation and/or the addition of diverse C_5 ribose-derived side chains (Flesch and Rohmer, 1988; Rohmer, 1993). To date, few studies have successfully investigated the genes or proteins involved in modifying the hopanoid core. HpnP and HpnR have been identified as the radical S-adenosylmethionine (SAM) enzymes responsible for production of 2- and 3-methylhopanoids, respectively (Welander et al., 2010; Welander and Summons, 2012). The production of C-3 methylated hopanoids is important for late stationary survival and maintenance of intracytoplasmic membranes in *Methylococcus capsulatus* (Welander and Summons, 2012), while the production of C-2 methylated hopanoids is important for stress tolerance (Welander and Summons, 2012; Kulkarni et al., 2013). Hopanoids with C_{35} extended side chains are common among hopanoid producers, but little is known about their biosynthesis or their function(s). The B-12 binding radical SAM protein HpnH and the nucleoside phosphorylase HpnG catalyze the first and second steps in hopanoid side chain biosynthesis, respectively (Bradley et al., 2010; Welander et al., 2012b). In *Rhodopseudomonas palustris*, the deletion of *hpnH* resulted in a strain only capable of producing C_{30}

hopanoids, and with compromised integrity of the outer membrane (Welander et al., 2012b). These discoveries have provided much needed insight into the initiation of C_{35} hopanoid production; however, the additional enzymes involved in side chain biosynthesis and the specific role that different functionalized hopanoids play in membrane stability and stress tolerance remain to be elucidated.

Burkholderia cenocepacia is one of the 17 species of genetically related bacteria known as the *B. cepacia* complex (Bcc). These bacteria are widely spread in aquatic and soil environments where they play beneficial roles including the promotion of plant growth and the degradation of pollutants (Coenye and Vandamme, 2003). Bcc bacteria are also opportunistic pathogens, causing infections in cystic fibrosis patients and other immune-compromised individuals (Vandamme et al., 1997; Speert, 2002). Given their particular environmental niche, it is not surprising that *Burkholderia* species are intrinsically resistant to most clinically relevant antibiotics and antimicrobial peptides (Waters and Ratjen, 2006; Loutet and Valvano, 2011). Resistance to antimicrobials depends, in part, on the production of hopanoids (Schmerk et al., 2011). The deletion of both squalene-hopene cyclase (Shc) encoding genes in *B. cenocepacia* K56-2 resulted in a strain (Δ*shc*) that could not produce hopanoids and displayed increased sensitivity to low pH, detergent, and various antibiotics. The mutant was also unable to produce flagella, resulting in severely diminished motility (Schmerk et al., 2011). Several *Burkholderia* species produce various C_{35} extended hopanoids, including bacteriohopanetetrol (BHT), BHT glucosamine, and BHT cyclitol ether; however, the functional importance of these individual hopanoids has not been explored (Cvejic et al., 2000; Talbot et al., 2007b).

This study identifies the types of extended hopanoids produced by *B. cenocepacia* and utilizes genetic tools to generate a collection of mutants in predicted hopanoid biosynthetic genes. Environmental stress tests performed with the mutants lead to new suggestions about the biological roles of C_{35} extended hopanoids. Our results are the first to define the biosynthetic pathway of hopanoids in *Burkholderia* and to define a biosynthetic role for the proteins HpnI, HpnJ and HpnK in any hopanoid producing bacterium.

Results and Discussion

B. cenocepacia *produces a mixture of hopanoids*

The biosynthesis of hopanoids requires the cyclization of squalene, a reaction catalyzed by squalene-hopene cyclase (Ochs et al., 1992), followed by the functionalization of the basic hopanoid structure through the addition of various side chains and additional modifications. Previously (Schmerk et al., 2011), we reported the identification and characterization of two paralogs of the squalene-hopene cyclase gene (*shc*) in *B. cenocepacia*, which are located on chromosomes 2 (BCAM2831) and 3 (BCAS1067), herein designated as *hpnF2* and *hpnF3*, respectively (Fig. 2). GC analysis of lipid extracts obtained from the deletion mutant lacking both *hpnF* genes (Δ*shc*) demonstrated that this strain did not produce hopanoids but at the same time accumulated an unidentified compound, which was also produced by the *hpnF2* single deletion mutant (Schmerk et al., 2011). This compound was subsequently identified as squalene (Figure S1). We next examined lipid extracts from stationary phase liquid cultures to determine the species of functionalized hopanoids produced by *B. cenocepacia*. These experiments revealed that *B. cenocepacia* produces various hopanoid structures including the C_{30} hydrocarbon diploptene and three functionalized bacteriohopanepolyols: bacteriohopanetetrol (BHT), BHT cyclitol ether, and BHT glucosamine (Figure 1a and Figure S2). *B. cenocepacia* also produced an unsaturated hopanoid. The mass spectral data was insufficient to distinguish if it is an unsaturated BHT cyclitol ether or BHT glucosamine, and whether the unsaturation is at C-6 or C-11 positions of the ring system (Figure 1a). However, our findings agree with previous studies reporting that a strain of *B. cepacia* produces these same saturated hopanoid species and also a C-6 unsaturated BHT cyclitol ether (Cvejic et al., 2000; Talbot et al., 2007a).

Characterization of hopanoid biosynthesis genes in B. cenocepacia

The hopanoid biosynthesis associated genes of *B. cenocepacia* are located in one cluster on chromosome 1 and two clusters on chromosome 2 (Figure 1b). In addition, the second squalene-hopene cyclase gene (*hpnF3*) is present as a single gene on chromosome 3, as discussed above. Homologs of *ispH/lytB* (BCAM2738 and BCAL2710), *hpnE* (BCAM2832) and *hpnC* (BCAM2833) are required for squalene production (Perzl et al., 1998; Wolff et al., 2003). $ispH_{BCAL2710}$ and $ispH_{BCAM2738}$ were deleted in a previous study (Loutet et al., 2011), while *hpnC* and *hpnE* could both

136 be essential for bacterial viability, as their homologs could not be deleted in *R. palustris* (Welander et
137 al., 2012b). In the present study, we focus on the genes required for the synthesis of the hopanoid
138 chain (Table 1).
139 The *hpnA* homolog (BCAM2735) could not be deleted; repeated attempts to delete this gene
140 resulted in exconjugants reverting to the wild type background. To confirm whether this gene was
141 essential for bacterial viability, we created a conditional mutant by placing *hpnA* under the control of
142 a rhamnose-inducible promoter (Ortega et al., 2007). The conditional P_{rhaB}::*hpnA* mutant could grow
143 in the presence of rhamnose (permissive condition) and glucose (non-permissive condition),
144 indicating that *hpnA* is not essential for *B. cenocepacia* (data not shown). The reasons why this gene
145 could not be deleted remain unknown. A similar case was encountered when attempting to delete
146 *hpnH* (BCAM2739). To successfully remove *hpnH* from the chromosome the deletion had to be
147 constructed in a Δ*shc* background strain (Schmerk et al., 2011). After *hpnH* was successfully deleted,
148 hopanoid production was restored by introducing pBCAM2831, which encodes the HpnF2 squalene-
149 hopene cyclase.
150 Chemical analyses of the hopanoids produced by the deletion mutants were performed by gas
151 chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-
152 MS), and demonstrated that five of the ten genes play roles in the production of extended hopanoids
153 (Table 2). The other five genes did not demonstrate a clear role in hopanoid biosynthesis. It is possible
154 that these genes are involved in the regulation of hopanoid production or their transport to specific
155 locations within the bacterial cell envelope, both of which were not evaluated in this study.
156
157 *HpnI and HpnK are required for bacteriohopanetetrol glucosamine production*
158 The B-12 binding radical SAM protein HpnH is responsible for the primary step in producing
159 extended hopanoids (Bradley et al., 2010; Welander et al., 2012b). The C_5 unit of the extended
160 hopanoid side chain is derived from a ribosugar, whose addition is catalyzed by HpnH (Figure 2)
161 (Bradley et al., 2010). The deletion of *hpnH* (BCAM2739) in *B. cenocepacia* resulted in a similar
162 effect to that observed in *M. extorquens* and *R. palustris*, with only the C_{30} hopanoid diploptene being
163 produced (Figure S3). Deletion of *hpnG* also prevented the production of any functionalized

hopanoids and LC-MS analysis demonstrated the sole accumulation of adenosyl hopane (Figure S4, Figure 2, step 2), an intermediate also found accumulated in *R. palustris* and *M. extorquens hpnG* mutants (Bradley et al., 2010; Welander et al., 2012b). With the adenine cleaved off the hopanoid compound by the nucleosidase HpnG (Figure 2, step 3), the resulting intermediate would be free to form bacteriohopanetetrol (BHT; Figure 2, step 4). In earlier studies, the final BHT intermediate has been suggested to be ribosyl hopane or derivatives such as ribonyl hopane and formyl hopane, and also phosphoribohopane rapidly cleaved to form BHT (Duvold and Rohmer, 1999; Seemann et al., 1999; Bradley et al., 2010; Welander et al., 2012b). However, the hopanoid biosynthesis associated gene clusters of *B. cenocepacia* do not encode any enzymes that could catalyze the formation of BHT from such intermediates, despite the common belief that one more enzyme is required for its production. Because monosaccharides such as ribose and glucose naturally exist in both cyclic and open chain forms, it is conceivable that the ribose opens up without the aid of an enzyme (Bradley et al., 2010). Therefore, we propose that the conversion of the ribose hopanoid to BHT occurs nonenzymatically.

LC-MS results revealed that the next enzyme for the formation of extended hopanoids is the glycosyltransferase HpnI (Figure 2, step 5, and Figure 3b). Labelled sugar incorporation experiments previously performed in *Zymomonas mobilis* demonstrated that the cyclopentane group added to the extended hopanoid side chain is derived from *N*-acetyl-D-glucosamine (GlcNAc) (Vincent et al., 2003). Bradley *et al*. have proposed that HpnI catalyzes the addition of GlcNAc to the hopanoid side chain (Bradley et al., 2010). Deletion of *hpnI* (BCAL1050) resulted in a mutant that only produced BHT (Figure 3b). However, it is still uncertain whether this enzyme utilizes the ribosylhopane intermediate or BHT as its substrate.

A deacetylation step (Figure 2, step 6), prior to ring contraction, is predicted to follow the glycosylation of the hopanoid side chain (Vincent et al., 2003). HpnK (BCAL1052) contains a YdjC protein motif which is thought to be involved in cellulose metabolism (Lai and Ingram, 1993). In *Citrobacter rodentium* the *hpnK* homolog is annotated as a chitobiose-phosphate hydrolase (*chbG*). A study of *chbG* in *E. coli* demonstrates that this gene encodes a monodeacetylase that acts on chitooligosaccharide substrates similar in structure to acetylglucosamine (Verma and Mahadevan,

2012). BLASTp analysis of *E. coli* ChbG against the *Burkholderia cenocepacia* J2315 protein database identifies HpnK as the polypeptide providing the most significant alignment (E-value $4e^{-15}$). Deletion of *hpnK* in *B. cenocepacia* resulted in production of BHT glucosamine but not BHT cyclitol ether (Figure 3c), indicating deacetylation is required for the subsequent production of BHT cyclitol ether. Because all lipid samples have to be acetylated as part of the LC-MS analysis protocol we were not able to directly detect the deacetylated BHT glucosamine.

HpnJ is essential for the production of bacteriohopanetetrol cyclitol ether

Deletion of *hpnJ* (BCAL1051) resulted in the loss of BHT cyclitol ether production while the production of the other extended hopanoids was maintained (Figure 3d). A ring contraction may be necessary to produce BHT cyclitol ether from BHT glucosamine (Figure 2, step 7), but the exact mechanism of the ring contraction reaction remains unknown (Vincent et al., 2003; Pan and Vincent, 2008). Like HpnH, HpnJ is annotated as a radical SAM protein. This family of enzymes catalyzes a wide range of reactions including RNA modifications and the synthesis of cofactors and antibiotics (Sofia et al., 2001), and utilize an enzyme-bound [4Fe–4S] cluster (Frey et al., 2008; Duschene et al., 2009; Shisler and Broderick, 2012). The iron-sulfur cluster is active in its reduced state and from this state it can transfer an electron to the sulfonium of SAM. This electron transfer promotes the homolytic cleavage of SAM, producing methionine and a 5'-deoxyadenosyl (5′-dAdo) radical intermediate (Nicolet et al., 2009). This highly reactive radical can abstract a hydrogen atom from its substrate, often from unreactive positions (Hioe and Zipse, 2012). Of the thousands of predicted radical SAM enzymes only a small number have been biochemically characterized (Sofia et al., 2001; Frey et al., 2008). However, a recent study provided mechanistic details for ring contraction by QueE, the radical SAM enzyme of *Bacillus subtilis* (McCarty et al., 2013). QueE utilizes SAM to abstract a hydrogen atom from 6-carboxy-5,6,7,8-tetrahydropterin (CPH_4) and perform a ring contraction similar to that observed in the conversion of BHT glucosamine to BHT cyclitol ether (McCarty et al., 2013).Therefore, it is possible that the ring contraction that converts the BHT glucosamine to the cyclitol ether also occurs through a radical SAM mechanism.

Phylogenetic analysis of HpnI and HpnJ homologs

The production of BHT glucosamine has been documented in several species, including *Z. mobilis*, *Geobacter sulfurreducens*, *G. metallireducens* and *B. cepacia* (Flesch and Rohmer, 1989; Talbot et al., 2007b; Eickhoff et al., 2013). Since the enzyme responsible for the synthesis of this hopanoid has been identified, bioinformatics analysis can be used to predict which bacteria could produce BHT glucosamine. An unrooted maximum likelihood tree was created using the top 250 sequences retrieved *via* a protein BLAST search of the *B. cenocepacia* HpnI sequence against the KEGG and NCBI databases (Figure 4). All bacteria contained within this tree encode at least one copy of the squalene-hopene cyclase HpnF, indicating that all of these strains have the capacity to produce hopanoids. The ability to produce BHT glucosamine appears to be widespread among hopanoid producers, with the gene encoding HpnI being present in α-, β-, δ-, and γ-proteobacterial species, as well as in various cyanobacteria and the poorly characterized phylum of acidobacteria. The bacterial taxa in this tree reside in a wide range of water and soil associated environments and must endure variations in temperature, pH and exposure to a variety of chemical and metal stresses (Diels et al., 2009; Loutet and Valvano, 2010; Roger et al., 2012; Mamlouk and Gullo, 2013). It is possible that complex extended hopanoids, like BHT glucosamine are important in maintaining bacterial membrane stability in these constantly changing environments. Most *Burkholderia* species, including *B. cenocepacia*, contain only one copy of *hpnI*, found within the beta proteobacteria clade with the closely related *Ralstonia* and *Cupriavidus* spp. However, several *Burkholderia* species, including *B. pseudomallei* and *B. thailandensis*, contain 2 copies of *hpnI*. This second copy of the gene lies within an alpha proteobacterial clade and may have been acquired through horizontal gene transfer. *Hyphomicrobium* spp. and *Phaeospirillum molischianum* also contain 2 copies of *hpnI* which are found within clades belonging to 2 different phyla, indicating that they too have likely acquired an extra copy of the gene via horizontal gene transfer.

Many bacterial taxa, including *M. fujisawaense* (Talbot et al., 2007b), *M. extoquorens* (Bradley et al., 2010), *G. sulfurreducens*, *G. metallireducens* (Eickhoff et al., 2013), *B. pseudomallei*, *B. gladioli*, *B. cepacia* (Cvejic et al., 2000; Talbot et al., 2007b) and *Candidatus Chloracidobacterium thermophilum* (Costas et al., 2012) produce BHT cyclitol ether. We created a maximum likelihood

248 tree of the top 250 sequences retrieved via a protein BLAST search of the *B. cenocepacia* HpnJ
249 sequence against the KEGG and NCBI databases (Figure 5). As mentioned previously, HpnJ is a
250 radical SAM protein and BLAST searches of radical SAM hopanoid biosynthesis proteins generally
251 pick up other radical SAM proteins that are not associated with hopanoid biosynthesis. To
252 differentiate between these non-hopanoid biosynthesis radical SAM proteins and true HpnJ homologs
253 the e-value for a bona fide HpnJ was set to e^{-100} or lower, as these was the lowest e-value for which a
254 homolog of known but different function could be identified. The majority of HpnJ homologs were
255 found in species that also contained an HpnI homolog, thereby suggesting that the production of BHT
256 cyclitol ether depends on the production of a BHT glucosamine precursor, as we have observed in *B.*
257 *cenocepacia*. As *B. cenocepacia* appears to produce much more BHT cyclitol ether than BHT
258 glucosamine (Figure 3a), we speculate that the former plays the dominant role in maintaining
259 membrane stability in response to environmental stresses.
260
261 *Extended hopanoids are important in protecting* B. cenocepacia *from environmental stresses*
262 Our previous work (Schmerk et al., 2011) demonstrated that hopanoid production plays an important
263 role in the ability of *B. cenocepacia* to grow under diverse stress conditions, including low pH, the
264 detergent sodium dodecyl sulfate (SDS), and the antimicrobial lipopeptide polymyxin B. This is likely
265 due to the capacity of hopanoids to maintain membrane stability, a notion that was consistent with the
266 observed retraction of the inner membrane from the outer membrane in the Δ*shc* strain, and the
267 mutant's inability to produce flagella (Schmerk et al., 2011). To determine whether extended
268 hopanoids play any role in the ability of *B. cenocepacia* to resist stress conditions we monitored the
269 growth of all mutants over a period of 24 h in LB buffered to pH 7.0 or pH 4.0, as well as LB
270 supplemented with 0.03% SDS or 1 mg ml^{-1} polymyxin B. Δ*hpnH*, which only produces the C_{30}
271 hopanoid diploptene, behaved like Δ*shc*, as it was unable to grow in all conditions tested, except in
272 the pH 7.0 control medium (Figure 6). Therefore, we conclude that the production of diploptene alone
273 is not sufficient for *B. cenocepacia* to fully adapt to the stress conditions tested. The susceptibility of
274 Δ*hpnH* to both SDS and polymyxin B indicates that this strain likely suffers from increased
275 membrane permeability, as increased sensitivity to detergents and antibiotics are indicators of

membrane damage in Gram-negative bacteria (Begley et al., 2005; Ruiz et al., 2005; Welander et al., 2009; Loutet and Valvano, 2011). We were unable to determine if this membrane damage results in decreased motility, as the construction of the *hpnH* strain required the presence of a complementing plasmid expressing *hpnF2* (pBCAM2831). The pSCRhaB2 vector used for this complementation severely alters motility patterns observed in swarming and swimming assays. There was no significant motility defect observed for any of the other nine mutants tested indicating that diploptene and/or adenosyl hopane, which is produced by Δ*hpnG*, alone can confer sufficient membrane integrity or stability to properly assemble the flagellar apparatus.

The remaining mutants with defects in hopanoid side chain assembly demonstrated a range of phenotypes under stress (Figure 7). Δ*hpnG*, which only produces adenosylhopane, had a phenotype similar to Δ*hpnH* and was unable to grow in all conditions tested aside from the pH 7.0 control. The minimum inhibitory concentration (MIC) of polymyxin B for Δ*hpnG* was 64 μg ml^{-1} (data not shown), a significantly lower value than that of the wild type (>1024 μg ml^{-1}), and comparable to that of Δ*shc* (128 μg ml^{-1}). Therefore, lack of hopanoids (Δ*shc*) and production of adenosyl hopane (Δ*hpnG*) are detrimental to the bacterium. Introducing a functional *hpnG* gene by complementation with pHpnG restored growth to near wild type levels in the presence of 0.03% SDS, and partially restored growth in pH 4.0 and in the presence of polymyxin B (Figure S5). Since the hopanoid intermediate produced by Δ*hpnG* does not accumulate in the wild type, it is difficult to determine whether the phenotypes observed in the mutant are due to the lack of C_{35} extended hopanoids or to the build-up of adenosylhopane itself. It is also possible that adenosylhopane intermediate may be somehow mislocalized within the bacterial cell.

The Δ*hpnI*, Δ*hpnJ*, and Δ*hpnK* mutants could grow similarly to wild type in 0.03% SDS (Figure 7), indicating that the presence of the C_{35} extended hopanoid BHT is sufficient to confer a higher degree of membrane integrity than the C_{30} hopanoid diploptene. Δ*hpnI* and Δ*hpnJ* were partially resistant to low pH and polymyxin B, and grew slower than wild type (Figure 7). Comparatively, Δ*hpnJ* displayed a less severe phenotype than Δ*hpnI*, demonstrating that the three C_{35} extended hopanoids are likely to play unique roles in enhancing the membrane integrity of *B. cenocepacia*.

303 Complementation of the Δ*hpnI* and Δ*hpnJ* mutants with pHpnI and pHpnJ, respectively, restored
304 growth at pH 4.0 and in the presence of polymyxin B to wild type levels (Figure S5).
305 Δ*hpnK*, which is thought to produce only acetylated BHT glucosamine, grew more poorly than
306 Δ*hpnI* and Δ*hpnJ* at pH 4.0 and could not in 1 mg ml $^{-1}$ polymyxin B (Figure 7). The MIC value of
307 polymyxin B for Δ*hpnI*, Δ*hpnJ*, and Δ*hpnK* was 256 μg ml^{-1} (data not shown). The acetylated BHT
308 glucosamine detected in this mutant is a hopanoid intermediate that would not normally be produced
309 in the wild type strain. As proposed for the *hpnG* mutant, it is possible that this intermediate interferes
310 with proper transport or membrane localization causing an increase in membrane permeability when
311 compared to Δ*hpnI* and Δ*hpnJ* mutants. Complementation of Δ*hpnK via* pHpnK was able to partially
312 restore growth at pH 4.0 and restored growth to a level similar to wild type in the presence of
313 polymyxin B (Figure S5).
314 The remaining mutants tested displayed high variations in their degree of sensitivity to the tested
315 stress conditions (Figure S6 and Table 3). We speculate that since these genes do not play a detectable
316 role in hopanoid side chain biosynthesis they may be involved in the regulation and/or membrane
317 transport of hopanoid molecules. The BCAM2736 and *hpnB* (BCAM2737) genes are highly
318 conserved among *Burkholderia* species and other hopanoid producing bacteria; however, the deletion
319 of these genes did not result in any defect in hopanoid biosynthesis or the ability of these mutants to
320 tolerate membrane stress. *Burkholderia* species are highly adaptive to a wide range of ecological
321 niches (Coenye and Vandamme, 2003), including the ability to colonize various hosts (Loutet and
322 Valvano, 2010). Therefore, the conditions used in our experiments most likely underestimate the full
323 spectrum of situations for which hopanoid production by *B. cenocepacia* could be required, and it is
324 possible that these genes may be required in situations not modeled by our experiments. There is little
325 information concerning the possible function of HpnL (BCAL1053). The loss of this protein resulted
326 in an intermediate phenotype, being able to grow as well as MH1K in the pH 7.0 buffered control and
327 in 0.03% SDS, but exhibiting delayed growth in both low pH medium and medium containing
328 polymyxin B (Figure S6).
329 Δ*hpnM* (BCAM2827) grew slower than MH1K at pH 4.0, and was unable to grow in the
330 presence of both detergent and polymyxin B (Figure S6). HpnM proteins are members of the toluene

tolerance protein Ttg2D family, a group of ABC-like transporters. Ttg2 plays a role in toluene tolerance in *Pseudomonas putida* (Kim et al., 1998), a process that involves increasing the cell membrane rigidity by changing the fatty acid and phospholipid compositions of the outer membrane (Ramos et al., 1997). MlaC is also a member of this protein family and is involved in maintaining lipid asymmetry via the retrograde trafficking of phospholipids from the outer to inner membrane in *E. coli* (Malinverni and Silhavy, 2009). It is likely that HpnM is also involved in the trafficking of lipids, specifically the glucosamine or cyclitol ether hopanoids, in response to environmental cues. HpnN is an RND-family transporter protein shown to be essential in the transport of hopanoids from the inner to outer membrane of *R. palustris* (Doughty et al., 2011). In our previous work, deletion of the *hpnN* gene in *B. cenocepacia* did not result in increased sensitivity to growth in low pH, SDS or polymyxin B but did result in increased sensitivity to other antibiotics (Schmerk et al., 2011). Together these results suggest that multiple transporter proteins may be involved in coordinating the trafficking of different hopanoids within the membrane of *B. cenocepacia* and likely other hopanoid producing bacteria.

Conclusions

In this study, we have defined the majority of the genes involved in the hopanoid biosynthetic pathway of *B. cenocepacia*. This information will illuminate future identification of the unique and specific functions that C_{35} extended hopanoids, such as BHT cyclitol ether, play in bacterial membrane physiology. Given that BHT provides a much higher degree of membrane integrity than diploptene, it is clear that C_{35} extended hopanoids, even in their most basic form, play a vital role in the function of the *B. cenocepacia* membrane. Identifying the genes responsible for the modification of extended hopanoids has also provided the tools needed to predict their structures based on genomic and metagenomic sequence information, and will help with the interpretation of geomicrobiological data. This work will also lead the way for future studies of functionalized hopanoids, providing insight into their specific biological functions while also allowing for a more informed interpretation of the hopanoid fossil record. Furthermore, the study of hopanoids in *B. cenocepacia* provides an opportunity to explore novel treatment options for cystic fibrosis patients infected with Bcc species.

As these species are intrinsically resistant to most clinically relevant antibiotics, the increased antibiotic sensitivity of the various hopanoid mutants raises the possibility of utilizing unique inhibitors, in combination with current antibiotic treatments, to better control infection in these patients.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in supplemental Table S1. Bacteria grew on LB agar plates or in LB broth with shaking at 37ºC. When necessary, *Escherichia coli* cultures were supplemented with 40 μg ml^{-1} kanamycin, and 30 μg ml^{-1} tetracycline. *B. cenocepacia* cultures were supplemented, as needed, with 100 μg ml^{-1} trimethoprim, and 150 μg ml^{-1} tetracycline.

Construction of deletion strains and complementing plasmids

The construction of unmarked, nonpolar mutant strains was performed as previously described by Flannagan *et al.* (Flannagan et al., 2008). The deletion mutagenesis plasmids were created by cloning ~600-800-bp DNA amplicons flanking each of the putative hopanoid biosynthesis associated genes. Amplified fragments were ligated into pGPI-SceI to create the desired deletion plasmids. The mutagenic plasmids were mobilized into *B. cenocepacia* MH1K by triparental mating and cointegrants selected using 100 μg ml^{-1} trimethoprim. Selection against *E. coli* donor and helper strains after the triparental mating was accomplished using 200 μg ml^{-1} ampicillin in combination with 25 μg ml^{-1} polymyxin B. The pDAI-SceI-SacB vector was used in the final stage of mutagenesis to induce the second recombination event, leading to an unmarked gene deletion. This vector was mobilized into *B. cenocepacia* MH1K cointegrants, and exconjugants were selected with 150 μg ml^{-1} tetracycline. Colonies were screened by PCR to confirm the presence of the appropriate gene deletions.

Complementing plasmids were constructed by amplifying *hpnG* (BCAM2830), *hpnI* (BCAL1050) and *hpnJ* (BCAL1051), and *hpnK* (BCAL1052) with the appropriate primer pairs. PCR products were

386 cloned into pSCrhaB2, resulting in the creation of pHpnG, pHpnI, pHpnJ, and pHpnK.
387 Complementing plasmids were introduced into the desired mutant strains by triparental mating as
388 described above. For unknown reasons, deletion of *hpnH* (BCAM2739) was not possible using the
389 method described above. To delete this gene the plasmid pDelBCAM2739 was mobilized into the
390 non-hopanoid-producing strain MH1KΔ*shc* (Schmerk et al., 2011), and the Δ*hpnH* mutant strain was
391 then created as outlined above. To restore hopanoid production following *hpnH* deletion, the *hpnF2*
392 gene (BCAM2831) was introduced via the complementing plasmid pBCAM2831 (Schmerk et al.,
393 2011).
394
395 *Analysis of hopanoids*
396 Lipid extracts from the wild type (MH1K) and mutant strains were prepared using the method of
397 Welander *et al.* (Welander et al., 2012b). Briefly, 200 ml of stationary phase culture were harvested
398 by centrifugation at 5000*g* for 10 min at 4ºC. Cells were disrupted by sonication in 10 ml of 10:5:4
399 (v:v:v) methanol (MeOH):dichloromethane (DCM):water for 15 min. Samples were centrifuged at
400 3000*g* for 10 min, the supernatant was transferred to a new tube and the pellet was treated once more.
401 Combined supernatants were separated into two phases via the addition of 10 ml DCM and 5 ml water
402 followed by centrifugation at 3000*g* for 10 min. The organic phase was placed in a new tube and the
403 residual aqueous phase was treated once more with 10 ml DCM and 5 ml water. Following
404 centrifugation, the organic phases were combined and evaporated under a stream of N_2 gas and the
405 total lipid extracts (TLE) were then dissolved in 2 ml DCM. To identify the production of the C_{30}
406 hopene, acetylated TLEs from each strain were analyzed by high temperature gas chromatography-
407 mass spectrometry (GC-MS) as previously described (Welander et al., 2009). Acetylated TLEs were
408 also analyzed by liquid chromatography-mass spectrometry (LC-MS) to identify any functionalized
409 hopanoids (Welander et al., 2012a). Details of the chromatographic analysis can be found in the
410 supplementary information.
411
412 *Phylogenetic analysis*

Homologs of *B. cenocepacia* HpnI and HpnJ were identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and National Center for Biotechnology Information (NCBI) databases via TBLASTN (Altschul et al., 1997) and the top 250 sequences were aligned via clustalW2 using the Geneious R6 software version 6.1.2 (Biomatters Ltd., Auckland, New Zealand, http://www.geneious.com/). Maximum likelihood trees were constructed by PhyML (http://www.atgc-montpellier.fr/phyml/) (Guindon et al., 2010) using the LG+gamma model, six gamma rate categories, ten random starting trees, SPR+NNI branch swapping, and substitution parameters estimated from the data. The finalized trees were generated by importing the resulting PhyML tree into iTOL for editing (http://itol.embl.de/) (Letunic and Bork, 2011).

Environmental stress tests

Strains grew overnight with shaking in unbuffered LB medium at 37ºC. Cultures were adjusted to an OD_{600} 0.005 in the appropriate medium. Buffered LB medium was prepared by adding 100mM (final concentration) MES (4-morpholineethanosulfonic acid) for pH 4.0 or 100mM MOPS (4-morpholionepropanesulfonic acid) for pH 7.0. Where appropriate, the pH 7.0 buffered LB medium was supplemented with 0.03% SDS (w/v) or 1 mg ml^{-1} polymyxin B. Growth was determined in a 100-well disposable plate using a Bioscreen C automated microbiology growth curve analysis system (MTX Lab Systems). Growth was monitored over 24 h at 37ºC.

Acknowledgements

We thank Florence Schubotz and Emily Matys for running the lipid samples for GC-MS and LC-MS analysis. This work was supported by grants from Cystic Fibrosis Canada (to M.A.V.) and from the Natural Sciences and Engineering Research Council of Canada (to M.A.B.). C.L.S. was supported by a postdoctoral fellowship from Cystic Fibrosis Canada. Work at MIT and Stanford was conducted with the support of awards from NSF (EAR-1147755) and the NASA Astrobiology Institute (NASA-NNA13AA90A).

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Begley, M., Gahan, C.G., and Hill, C. (2005) The interaction between bacteria and bile. *FEMS Microbiol Rev* **29**: 625-651.

Bradley, A.S., Pearson, A., Saenz, J.P., and Marx, C.J. (2010) Adenosylhopane: The first intermediate in hopanoid side chain biosynthesis. *Organic Geochemistry* **41**: 1075-1081.

Brocks, J.J., Love, G.D., Summons, R.E., and Logan, G.A. (2004) Purple sulfur bacteria in an intensely stratified Paleoproterozoic sea. *Geochimica Et Cosmochimica Acta* **68**: A796-A796.

Coenye, T., and Vandamme, P. (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environmental Microbiology* **5**: 719-729.

Costas, A.M.G., Tsukatani, Y., Rijpstra, W.I.C., Schouten, S., Welander, P.V., Summons, R.E., and Bryant, D.A. (2012) Identification of the Bacteriochlorophylls, Carotenoids, Quinones, Lipids, and Hopanoids of "*Candidatus Chloracidobacterium thermophilum*". *Journal of Bacteriology* **194**: 1158-1168.

Cvejic, J.H., Putra, S.R., El-Beltagy, A., Hattori, R., Hattori, T., and Rohmer, M. (2000) Bacterial triterpenoids of the hopane series as biomarkers for the chemotaxonomy of *Burkholderia*, *Pseudomonas* and *Ralstonia* spp. *FEMS Microbiol Lett* **183**: 295-299.

Diels, L., Van Roy, S., Taghavi, S., and Van Houdt, R. (2009) From industrial sites to environmental applications with *Cupriavidus metallidurans*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **96**: 247-258.

Doughty, D.M., Hunter, R.C., Summons, R.E., and Newman, D.K. (2009) 2-Methylhopanoids are maximally produced in akinetes of *Nostoc punctiforme*: geobiological implications. *Geobiology* **7**: 524-532.

Doughty, D.M., Coleman, M.L., Hunter, R.C., Sessions, A.L., Summons, R.E., and Newman, D.K. (2011) The RND-family transporter, HpnN, is required for hopanoid localization to the outer membrane of *Rhodopseudomonas palustris* TIE-1. *Proceedings of the National Academy of Sciences of the United States of America* **108**: E1045-E1051.

Duschene, K.S., Veneziano, S.E., Silver, S.C., and Broderick, J.B. (2009) Control of radical chemistry in the AdoMet radical enzymes. *Current Opinion in Chemical Biology* **13**: 74-83.

Duvold, T., and Rohmer, M. (1999) Synthesis of ribosylhopane, the putative biosynthetic precursor of bacterial triterpenoids of the hopane series. *Tetrahedron* **55**: 9847-9858.

Eickhoff, M., Birgel, D., Talbot, H.M., Peckmann, J., and Kappler, A. (2013) Bacteriohopanoid inventory of *Geobacter sulfurreducens* and *Geobacter metallireducens*. *Organic Geochemistry* **58**: 107-114.

Flannagan, R.S., Linn, T., and Valvano, M.A. (2008) A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ Microbiol* **10**: 1652-1660.

Flesch, G., and Rohmer, M. (1988) Prokaryotic hopanoids: the biosynthesis of the bacteriohopane skeleton. Formation of isoprenic units from two distinct acetate pools and a novel type of carbon/carbon linkage between a triterpene and D-ribose. *Eur J Biochem* **175**: 405-411.

Flesch, G., and Rohmer, M. (1989) Prokaryotic triterpenoids. A novel hopanoid from the ethanol-producing bacterium *Zymomonas mobilis*. *Biochem J* **262**: 673-675.

Frey, P.A., Hegeman, A.D., and Ruzicka, F.J. (2008) The radical SAM superfamily. *Critical Reviews in Biochemistry and Molecular Biology* **43**: 63-88.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010) New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* **59**: 307-321.

Hioe, J., and Zipse, H. (2012) Hydrogen Transfer in SAM-Mediated Enzymatic Radical Reactions. *Chemistry-a European Journal* **18**: 16463-16472.

Kannenberg, E.L., and K. Poralla (1999) Hopanoid biosynthesis and function in bacteria. *Naurwissenschaften* **86**: 168-176.
Kim, K., Lee, S.J., Lee, K.H., and Lim, D.B. (1998) Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. *Journal of Bacteriology* **180**: 3692-3696.
Kulkarni, G., Wu, C.H., and Newman, D.K. (2013) The General Stress Response Factor EcfG Regulates Expression of the C-2 Hopanoid Methylase HpnP in *Rhodopseudomonas palustris* TIE-1. *Journal of Bacteriology* **195**: 2490-2498.
Lai, X.K., and Ingram, L.O. (1993) Cloning and Sequencing of a Cellobiose Phosphotransferase System Operon from *Bacillus stearothermophilus* Xl-65-6 and Functional Expression in *Escherichia coli*. *Journal of Bacteriology* **175**: 6441-6450.
Letunic, I., and Bork, P. (2011) Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Research* **39**: W475-W478.
Loutet, S.A., and Valvano, M.A. (2010) A decade of *Burkholderia cenocepacia* virulence determinant research. *Infect Immun* **78**: 4088-4100.
Loutet, S.A., and Valvano, M.A. (2011) Extreme antimicrobial peptide and polymyxin B resistance in the genus *Burkholderia*. *Front Cell Infect Microbiol* **1**: 6.
Loutet, S.A., Mussen, L.E., Flannagan, R.S., and Valvano, M.A. (2011) A two-tier model of polymyxin B resistance in *Burkholderia cenocepacia*. *Environ Microbiol Rep* **3**: 278-285.
Malinverni, J.C., and Silhavy, T.J. (2009) An ABC transport system that maintains lipid asymmetry in the Gram-negative outer membrane. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 8009-8014.
Malott, R.J., Steen-Kinnaird, B.R., Lee, T.D., and Speert, D.P. (2012) Identification of Hopanoid Biosynthesis Genes Involved in Polymyxin Resistance in *Burkholderia multivorans*. *Antimicrobial Agents and Chemotherapy* **56**: 464-471.
Mamlouk, D., and Gullo, M. (2013) Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. *Indian Journal of Microbiology* **53**: 377-384.
McCarty, R.M., Krebs, C., and Bandarian, V. (2013) Spectroscopic, Steady-State Kinetic, and Mechanistic Characterization of the Radical SAM Enzyme QueE, Which Catalyzes a Complex Cyclization Reaction in the Biosynthesis of 7-Deazapurines. *Biochemistry* **52**: 188-198.
Nicolet, Y., Amara, P., Mouesca, J.M., and Fontecilla-Camps, J.C. (2009) Unexpected electron transfer mechanism upon AdoMet cleavage in radical SAM proteins. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 14867-14871.
Ochs, D., Kaletta, C., Entian, K.D., Beck-Sickinger, A., and Poralla, K. (1992) Cloning, expression, and sequencing of squalene-hopene cyclase, a key enzyme in triterpenoid metabolism. *J Bacteriol* **174**: 298-302.
Ortega, X.P., Cardona, S.T., Brown, A.R., Loutet, S.A., Flannagan, R.S., Campopiano, D.J. et al. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. *J Bacteriol* **189**: 3639-3644.
Ourisson, G., and Albrecht, P. (1992) Hopanoids .1. Geohapanoids - the Most Abundant Natural-Products on Earth. *Accounts of Chemical Research* **25**: 398-402.
Ourisson, G., Rohmer, M., and Poralla, K. (1987) Prokaryotic hopanoids and other polyterpenoid sterol surrogates. *Annu Rev Microbiol* **41**: 301-333.
Pan, W.D., and Vincent, S.P. (2008) Synthesis of a deuterated analogue of bacteriohopanetetrol-glucosamine, a probe of complex hopanoid biosynthesis. *Organic & Biomolecular Chemistry* **6**: 2394-2399.
Perzl, M., Reipen, I.G., Schmitz, S., Poralla, K., Sahm, H., Sprenger, G.A., and Kannenberg, E.L. (1998) Cloning of conserved genes from *Zymomonas mobilis* and *Bradyrhizobium japonicum* that function in the biosynthesis of hopanoid lipids. *Biochim Biophys Acta* **1393**: 108-118.
Ramos, J.L., Duque, E., RodriguezHerva, J.J., Godoy, P., Haidour, A., Reyes, F., and FernandezBarrero, A. (1997) Mechanisms for solvent tolerance in bacteria. *Journal of Biological Chemistry* **272**: 3887-3890.
Roger, M., Castelle, C., Guiral, M., Infossi, P., Lojou, E., Giudici-Orticoni, M.T., and Ilbert, M. (2012) Mineral respiration under extreme acidic conditions: from a supramolecular organization to a

545 molecular adaptation in *Acidithiobacillus ferrooxidans*. *Biochemical Society Transactions* **40**: 1324-
546 1329.
547 Rohmer, M. (1993) The Biosynthesis of Triterpenoids of the Hopane Series in the Eubacteria - a Mine
548 of New Enzyme-Reactions. *Pure and Applied Chemistry* **65**: 1293-1298.
549 Rohmer, M., Bouvier-Navé, P., and Ourisson, G. (1984) Distribution of hopanoid tripertenes in
550 prokaryotes. *J Gen Microbiol* **130**: 1137-1150.
551 Ruiz, N., Falcone, B., Kahne, D., and Silhavy, T.J. (2005) Chemical conditionality: a genetic strategy
552 to probe organelle assembly. *Cell* **121**: 307-317.
553 Schmerk, C.L., Bernards, M.A., and Valvano, M.A. (2011) Hopanoid Production Is Required for
554 Low-pH Tolerance, Antimicrobial Resistance, and Motility in *Burkholderia cenocepacia*. *Journal of*
555 *Bacteriology* **193**: 6712-6723.
556 Seemann, M., Bisseret, P., Tritz, J.P., Hooper, A.B., and Rohmer, M. (1999) Novel bacterial
557 triterpenoids of the hopane series from *Nitrosomonas europaea* and their significance for the
558 formation of the C-35 bacteriohopane skeleton. *Tetrahedron Letters* **40**: 1681-1684.
559 Shisler, K.A., and Broderick, J.B. (2012) Emerging themes in radical SAM chemistry. *Current*
560 *Opinion in Structural Biology* **22**: 701-710.
561 Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F., and Miller, N.E. (2001) Radical SAM, a
562 novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical
563 mechanisms: functional characterization using new analysis and information visualization methods.
564 *Nucleic Acids Research* **29**: 1097-1106.
565 Speert, D.P. (2002) Advances in *Burkholderia cepacia* complex. *Paediatr Respir Rev* **3**: 230-235.
566 Summons, R.E., and Lincoln, S.A. (2012) Biomarkers: Informative molecules for studies in
567 geobiology. . In *Fundamentals of Geobiology*. Knoll, A.H., Canfield, D.E., and Konhauser, K.O.
568 (eds): Blackwell Publishing Ltd., pp. 269-296.
569 Talbot, H.M., Rohmer, M., and Farrimond, P. (2007a) Structural characterisation of unsaturated
570 bacterial hopanoids by atmospheric pressure chemical ionisation liquid chromatography/ion trap mass
571 spectrometry. *Rapid Communications in Mass Spectrometry* **21**: 1613-1622.
572 Talbot, H.M., Rohmer, M., and Farrimond, P. (2007b) Rapid structural elucidation of composite
573 bacterial hopanoids by atmospheric pressure chemical ionisation liquid chromatography/ion trap mass
574 spectrometry. *Rapid Communications in Mass Spectrometry* **21**: 880-892.
575 Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R. et al. (1997)
576 Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal
577 of *Burkholderia multivorans* sp. *nov*. *Int J Syst Bacteriol* **47**: 1188-1200.
578 Verma, S.C., and Mahadevan, S. (2012) The chbG Gene of the Chitobiose (chb) Operon of
579 *Escherichia coli* Encodes a Chitooligosaccharide Deacetylase. *Journal of Bacteriology* **194**: 4959-
580 4971.
581 Vincent, S.P., Sinay, P., and Rohmer, M. (2003) Composite hopanoid biosynthesis in *Zymomonas*
582 *mobilis*: N-acetyl-D-glucosamine as precursor for the cyclopentane ring linked to
583 bacteriohopanetetrol. *Chemical Communications*: 782-783.
584 Waters, V., and Ratjen, F. (2006) Multidrug-resistant organisms in cystic fibrosis: management and
585 infection-control issues. *Expert Rev Anti Infect Ther* **4**: 807-819.
586 Welander, P.V., and Summons, R.E. (2012) Discovery, taxonomic distribution, and phenotypic
587 characterization of a gene required for 3-methylhopanoid production. *Proceedings of the National*
588 *Academy of Sciences of the United States of America* **109**: 12905-12910.
589 Welander, P.V., Coleman, M.L., Sessions, A.L., Summons, R.E., and Newman, D.K. (2010)
590 Identification of a methylase required for 2-methylhopanoid production and implications for the
591 interpretation of sedimentary hopanes. *Proceedings of the National Academy of Sciences of the United*
592 *States of America* **107**: 8537-8542.
593 Welander, P.V., Doughty, D.M., Mehay, S., Summons, R.E., and Newman, D.K. (2012a)
594 Identification and characterization of *Rhodopseudomonas palustris* TIE-1 hopanoid biosynthesis
595 mutants. *Geobiology* **10**: 163-177.
596 Welander, P.V., Hunter, R.C., Zhang, L., Sessions, A.L., Summons, R.E., and Newman, D.K. (2009)
597 Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris*
598 TIE-1. *J Bacteriol* **191**: 6145-6156.

599 Welander, P.V., Doughty, D.M., Wu, C.H., Mehay, S., Summons, R.E., and Newman, D.K. (2012b)
600 Identification and characterization of *Rhodopseudomonas palustris* TIE-1 hopanoid biosynthesis
601 mutants. *Geobiology* **10**: 163-177.
602 Wolff, M., Seemann, M., Bui, B.T.S., Frapart, Y., Tritsch, D., Estrabot, A.G. et al. (2003) Isoprenoid
603 biosynthesis via the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl
604 diphosphate reductase (LytB/IspH) from *Escherichia coli* is a [4Fe-4S] protein. *Febs Letters* **541**:
605 115-120.

606

607

608 Table 1 Hopanoid biosynthesis genes deleted in *B. cenocepacia*

Gene name	Locus Tag	Annotation	Function	Reference
hpnI	***BCAL1050***	**Hopanoid-associated glycosyl transferase**	**Transfer of acetylglucosamine from UDP-acetylglucosamine to BHT**	This study
hpnJ	***BCAL1051***	**Hopanoid-associated radical SAM superfamily protein**	**Catalyzes ring contraction to generate BHT cyclitol ether**	This study
hpnK	***BCAL1052***	**Hopanoid biosynthesis associated protein (ydjC)**	**Deacetylation of BHT acetylglucosamine**	This study
hpnL	***BCAL1053***	**Conserved hypothetical protein**	**Not established**	
Unassigned	***BCAL1054***	**Putative lipoprotein (SmpA/OmlA)**	**Not established**	
*hpnA**	*BCAM2735*	Hopanoid-associated sugar epimerase	**Not established**	
Unassigned	***BCAM2736***	**Conserved hypothetical protein**	**Not established (only occurs in *Burkholderia* spp.)**	
hpnB	***BCAM2737***	**Hopanoid-associated glycosyl transferase**	**Not established**	
ispH/lytB	*BCAM2738*	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	**Not established**	
hpnH	***BCAM2739***	**Hopanoid-associated radical SAM superfamily protein**	**Addition of adenosine to hopane skeleton**	Bradley *et al.* 2010, Welander *et al.* 2012b, this study
hpnM	***BCAM2827***	**Hopanoid biosynthesis associated membrane protein**	**Not established**	
hpnN	*BCAM2828*	Hopanoid-associated RND transporter	Transport of hopanoids to the outer mmebrane	Doughty *et al.* 2011
hpnG	***BCAM2830***	**Hopanoid-associated nucleosidase**	**Removal of adenine from adenosyl hopane**	Bradley *et al.* 2010, Welander *et al.* 2012b, this study
hpnF2	*BCAM2831*	Squalene hopene cyclase	Cyclization of squalene	Reipen *et al.* 1995
hpnF3	*BCAS0167*	Squalene hopene cyclase	Cyclization of squalene	Reipen *et al.* 1995
hpnE	*BCAM2832*	Putative dehydrosqualene reductase	Production of squalene	Perzl *et al.* 1998
hpnC	*BCAM2833*	Squalene/phytoene synthase	Production of squalene	Perzl *et al.* 1998

609 Genes shown in bold were deleted in this study. *Unable to construct this deletion

610

Table 2. Hopanoids idneitifed in *B. cenocepacia* K56-2 mutants

	Diploptene	Bacterio hopanetetrol (BHT)	BHT cyclitol ether	Monounsaturated BHT cyclitol ether or glucosamine	BHT glucosamine	Adenosyl hopane
MH^+:	410	715	1002	1000	1002	746
Base Peak:	191	655	1002	1000	1002	611
Identification of these hopanoids in the following strains:						
K56-2	+	+	+	+	+	-
Δ*hpnF2* (BCAM2831)	+	+	+	+	+	-
Δ*hpnF3* (BCAS0167)	+	+	+	+	+	-
Δ*shc* BCAS0167, BCAM2831)	-	-	-	-	-	-
Δ*BCAM2736*	+	+	+	+	+	-
Δ*hpnB* (BACM2737)	+	+	+	+	+	-
Δ*hpnH* (BCAM2739)	+	-	-	-	-	-
Δ*hpnM* (BCAM2827)	+	+	+	+	+	-
Δ*hpnN* (BCAM2828)	+	+	+	+	+	-
Δ*hpnG* (BACM2830)	+	-	-	-	-	+
Δ*hpnI* (BCAL1050)	+	+	-	-	-	-
Δ*hpnJ* (BCAL1051)	+	+	-	-	+	-
Δ*hpnK* (BCAL1052)	+	-	-	-	+	-
Δ*hpnL* (BCAL1053)	+	+	+	+	+	-
Δ*BCAL1054* (*smpA/omlA*)	+	+	+	+	+	-

668 Table 3 Summary of resistance of hopanoid mutants to various environmental stresses

	Hopanoid side chain biosynthesis	Resistance to low pH stress	Resistance to SDS	Resistance to polymyxin B
Δ*hpnH* (BCAM2739)	+	+	+	+
Δ*hpnG* (BCAM2830)	+	+	+	+
Δ*hpnI* (BCAL1050)	+	+	-	+
Δ*hpnJ* (BCAL1051)	+	+	-	+
Δ*hpnK* (BCAL1052)	+	+	-	+
Δ*hpnM* (BCAM2827)	-	+	-	+
Δ*hpnL* (BCAL1053)	-	+	-	+
Δ*BCAM2736*	-	-	-	-
Δ*hpnB* (BCAM2737)	-	-	-	-
Δ*BCAL1054* (*smpA/omlA*)	-	+	+	+

669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699

Figures Legends

Figure 1 (**a**) Hopanoid compounds detected in *B. cenocepacia* K56-2 lipid extracts at stationary phase. The dotted rectangle contains the structures for C-6 and C-11 monounsaturated BHT cyclitol ethers; however, the mass spectra could not distinguish whether the BHT cyclitol ether or the BHT glucosamine is unsaturated and whether the unsaturation occurs at C-6 or C-11 ring positions. (**b**) The *B. cenocepacia* hopanoid biosynthetic gene clusters are present on chromosome 1, chromosome 2, and chromosome 3. Black arrows indicate genes that have been deleted in this study, white arrows indicate genes that were not deleted, and gray arrows indicate genes that were deleted in a previous study (Schmerk et al. 2011).

Figure 2 Proposed *B. cenocepacia* hopanoid biosynthesis pathway. Dotted arrows in step 8 represent a proposed desaturation reaction.

Figure 3 LC-MS extracted ion chromatograms of acetylated total lipid extracts from (**a**) wild type *B. cenocepacia*, (**b**) Δ*hpnI*, (**c**) Δ*hpnK*, and (**d**) Δ*hpnJ*. The chromatograms are a combination of ions *m/z* 1002 (I, BHT cyclitol ether and II, BHT glucosamine), 655 (III, bacteriohopanetetrol), and 1000 (IV, monounsaturated BHT cyclitol ether or glucosamine). Hopanoids were identified by comparison of their mass spectra to previously published samples (Talbot et al., 2007a; Talbot et al., 2007b; Talbot et al., 2003a; Talbot et al., 2003b).

Figure 4 Maximum likelihood phylogenetic tree of hopanoid associated glycosyl transferase, HpnI, among sequenced genomes. The *Methylobacterium* spp. and *Burkholderia* spp. (highlighted in red) clades have been collapsed due to the high number of strains present. Some *Burkholderia* species possess 2 copies of HpnI however *B. cenocepacia* strains contain only 1copy (found in the blue highlighted clade). All organisms present contain at least one copy of *shc* in their genome.

Figure 5 Maximum likelihood phylogenetic tree of hopanoid biosynthesis associated radical SAM protein, HpnJ. The *Methylobacterium* spp., *Cupriavidus* spp., and *Burkholderia* spp. (highlighted in red) clades have been collapsed due to the high number of strains present. With the exception of the collapsed outgroup clades, all organisms contain one or more copies of squalene hopene cyclase in their genome.

Figure 6 A *B. cenocepacia* Δ*hpnH* mutant exhibits sensitivity to environmental stresses. Representative growth curves of the wild type (MH1K) and mutant strain in LB buffered to pH 7.0 or pH 4, LB buffered to pH 7.0 supplemented with 0.03% SDS, and LB supplemented with 1 mg ml^{-1} polymyxin B. Δ*shc* is included as a control that cannot produce any hopanoids. All control strains contain the empty complementing vector pSCRhaB2. The Δ*hpnH* mutant had to be created in a Δ*shc* mutant background and contains pBCAM2831 to restore hopanoid biosynthesis. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.

Figure 7 Mutants involved in the biosynthesis of C_{35} extended hopanoid side chains display a range of sensitivity to environmental stresses. Representative growth curves of the wild type and mutant strains in LB buffered to pH 7.0, LB buffered to pH 4, LB buffered to pH 7.0 and supplemented with 0.03% SDS, and LB with 1mg ml^{-1} polymyxin B. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.

Supporting information

Supporting Information A. Hopanoids analyses

Table S1. Bacterial strain sand plasmids used in this study.

Table S2. Primers used in this study.

Figure S1 Identification of squalene build-up in squalene-hopene cyclase gene deletion mutants. Nonsaponifiable lipids were extracted from the *B. cenocepacia* Δ*hpnF2* strain and separated by GC. (**a**) Total ion chromatogram was compared to that of a squalene standard. (**b**) Full mass spectrum of the co-eluting peaks from panel (a). The Δ*shc* mutant (lacking both the *hpnF2* and *hpnF3* genes) accummulated an identical peak as in (a) (data not shown).

Figure S2. Mass spectra of functionalized hopanoids produced by wild type *B. cenocepacia* and hopanoid mutants. The top row contains the mass spectra of the hopanoid illustrated above. The bottom row shows the MS-MS spectra of the indicated ion. The unsaturated hopanoid could be either the cyclitol ether or glucosamine hopaniod; it is unclear from the mass spectra which functionalized hopanoid is unsaturated and where the unsaturation occurs (C-6 or C-11).

Figure S3. LC-MS and GC-MS analysis of *B. cenocepacia* Δ*hpnH* acetylated total lipid extract. (**a**) LC-MS combined extracted ion chromatogram (*m/z* 611, 655, 1000, 1002) demonstrating the lack of functionalized hopanoid production. (**b**) GC-MS extracted ion chromatogram (*m/z* 191) demonstrating the production of diploptene.

Figure S4. LC-MS analysis of *B. cenocepacia* Δ*hpnG* acetyalted total lipid extract. (**a**) Combined extracted ion chromatogram (*m/z* 611, 655, 1000, 1002) demonstrating the single adenosyl hopane peak at 29 minutes. (**b**) Mass spectra of the adenosyl hopane peak showing the 788 ion representing intact adenosyl hopane and the 611 ion representing the loss of an adenine molecule.

Figure S5. Complementation of the hopanoid side chain biosynthesis growth defects. Representative growth curves of the wild type (MH1K), mutant, and complement strains in LB buffered to pH 7.0, LB buffered to pH 4.0, LB buffered to pH 7.0 and supplemented with 0.03% SDS, and LB with 1mg ml^{-1} polymyxin B. Δ*shc* is included as a control that cannot produce any hopanoids. All control and mutant strains contain the empty complementing vector pSCRhaB2. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.

Figure S6. Mutants that are not directly involved in the biosynthesis of hopanoid side chains display an array of sensitivity to different environmental stresses. Representative growth curves of the wild type and mutant strains in LB buffered to pH 7.0, LB buffered to pH 4.0, LB buffered to pH 7.0 and supplemented with 0.03% SDS, and LB with 1 mg ml^{-1} polymyxin B. Each time point represents the average of three replicate cultures (the error bars represent standard deviations). Each growth curve was repeated at least three times.